CERTIFICATE OF MAILING

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0630/0D532

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

NEUHOLD et al.

Serial No.: 08/994,689 Group Art Unit: 1633

Filed: December 19, 1997 Examiner: M. Wilson

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

DECLARATION OF LISA A. NEUHOLD, Ph.D.,
UNDER C.F.R. § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

LISA A. NEUHOLD, Ph.D., declares and states that:

1. I am a co-inventor of the above-identified patent application.

2. I presently hold a position as Senior Research Scientist I, Molecular Genetics, for Wyeth-Ayerst Research, Princeton, New Jersey, which is a division of American

Home Products Corporation, assignee of this application and the invention.

3. My qualifications as a scientist are set forth on the copy of my *curriculum vitae* which is attached as Exhibit A.

4. I have read and am familiar with the Office Action dated November 6, 1998. I understand that claims 1-17 and 22-27 have been rejected for lack of enablement. It is my understanding that the Examiner believes that the specification does not teach how to make transgenics in any other species other than mice and that the teachings of this application fail to provide a correlation between the effects of mutations in MMP, specifically MMP13, and cartilage-related diseases. The following data, which have been obtained in accordance with the teachings of this application, further demonstrate that the transgenic animal model of the invention is an effective animal model that mimics human osteoarthritis and related pathological syndromes and that the promoters utilized are chondrocyte specific and functional in a number of different mammalian cells.

5. Tet-regulated synthetic MMP13 genes and expression constructs were prepared and tested as described in Examples 1 and 2 (pages 33-38 of the Specification).

6. In addition to verifying the MMP13* activity, prior to microinjection, both transgene constructs in Examples 1 and 2 of the specification were tested in primary bovine chondrocytes, primary chick chondrocytes, mouse embryonic fibroblasts and HeLa cells. The constructs were not expressed in either the mouse fibroblast or HeLa cells and showed strong expression in both the bovine and chick chondrocytes (data not shown). The results demonstrate the ability of this rat collagen II promoter to induce expression of a second construct containing

either the Tet07-luciferase or the Tet07-MMP13* in a broad range of chondrocytes. The rat promoter is expected to be active in mammalian rat chondrocytes, is shown to be active in both mammalian bovine chondrocytes and avian chick chondrocytes. In addition, the data described in the specification and below demonstrates its strong activity in mammalian mouse chondrocytes. Thus, the constructs of the invention are specifically active in both mammalian and avian chondrocytes and are thus shown to be useful in the generation of the non-human mammalian transgenic animals of the invention.

7. Exhibit B, which corresponds to Figure 3 in the application, dramatically demonstrates tissue-restricted expression of a transgene under control of a regulatory system for use in the invention. Exhibit B-A is a diagram of the construct, in which the rat type II collagen promoter drives expression of *LacZ*, which is followed by a β-globin splice and a polyadenylation signal. Exhibit B-B is a photographic illustration corresponding to Figure 3B in the application of whole mount staining for β-galactosidase activity of embryonic day 16 transgenic mouse embryos expressing the transgene (see Figure 3A in the application). Blue staining is evident in the joints throughout the body of the transgenic animal, while no staining is observed in the non-transgenic, wild-type littermate. Specifically, joints including the ankles, knees, hips, phalanges, wrists, elbows, shoulders, and vertebrae. In addition to the cartilage of the joints, cartilage that has not ossified to bone at this stage of development, *i.e.*, some of the facial, skull, and rib bones also stained blue. These data confirm the expression abilities of the type II collagen promoter, and are useful in determining those tissues (joints) that will be expressing our MMP13* transgene. Exhibit B-C shows an enlargement of the elbow and paw

(this figure corresponds to Figure 3C in the application). These color figures are provided to clearly demonstrate that transgene expression in this regulated system is limited to the joint, and accordingly is highly relevant to osteoarthritis. Color images demonstrate these data effectively and dramatically.

8. The constructs were co-microinjected into fertilized mouse embryos (see Example 3, pages 38-39 of the Specification). Out of 112 newborn mice, 7 transgenic founders harboring both transgenes were identified, however, only four of these transgenic lines were capable of breeding. The transgenes were identified by PCR and verified by Southern blot analysis using a transgene-specific probe (data not shown). The copy number for each of the 4 transgenics was further assessed using Taqman quantitative PCR (data not shown). Briefly, transgene copy number ranged from 1-32 and 1-20 for the tet activator and MMP13*, respectively. Specifically, line 6 contained ~8 copies of the tet activator and ~3 copies of the MMP13* transgene. The remainder of the data in this Declaration focuses on the expression analysis of line 6.

9. Expression of the TA and MMP13* transgenes were initially evaluated in the hind-knee joints of four-month old mice by PCR (see Example 3, pages 38-39 of the Specification). Amplification of the c-fos endogenous cDNA was used as a control to verify the efficacy of each reaction. Exhibit C-A shows amplification of an 890 bp fragment resulting from a TA-specific primer set. Reverse Transcriptase-PCR (RT-PCR) showed the TA transgene to be expressed in transgenic mice both on and off Dox, but was not expressed in the non-transgenic controls (lanes 4-5). The method used for RT-PCR is described in the specification at page 42,

lines 2-6 in Example 5. Constitutive expression of the TA is expected since it is driven by a constitutively active collagen type II promoter. Moreover, expression of the TA is limited to the joints and was not observed by RT-PCR in other tissues including brain, heart, liver, kidney, spleen, or skeletal muscle (data not shown).

10. Exhibit C-B shows amplification of a 645 bp fragment resulting from an MMP13* specific primer set. Note, the MMP13* primer set is specific for human MMP13 and does not react with it's endogenous mouse homologue, collagenase-1. RT-PCR showed that MMP13* was not expressed in the non-transgenic controls (lanes 4-5). Lanes 6-7 show that there is expression of the MMP13* transgene in mice maintained on Dox. Removal of Dox from the drinking water induces a significant amount of expression (lanes 8-9). We have estimated the expressed amount in this transgenic mouse line (line 6) to correspond to a 3-4 fold induction. This induction was estimated using RT-PCR and titrating the amount of MMP13* cDNA. Exhibit D shows that amplification of 2.0 ul of cDNA made from a transgenic on Dox is required to obtain the same level of signal from 0.5 ul of cDNA made from a trangenic off Dox. Furthermore, following gel electrophoresis, PCR fragments were transferred to a nylon membrane and hybridized to a TA or MMP13* specific probe to verify the identity of the PCR product (data not shown).

11. To access any changes in the articular cartilage due to transgene expression, mice from line 6 were maintained or removed from Dox for 114 days, and their joints were sectioned and stained with hematoxylin and eosin (H&E) (see page 19, lines 4-6 of the Specification). When compared with an age matched littermate control, the transgenic removed

from Dox developed a pathology reminiscent of osteoarthritis (Exhibit E-B). The control animal showed no lesions or other osteoarthritis pathologies (Exhibit E-A), whereas the transgenic animal shows the formation of lesions in its articular cartilage (Exhibit E-B). More specifically, the H&E sections show considerable loss of cartilage, focal erosions, erosions that extend into the bone, and an inflamed synovium. Within the synovium there is evidence of fibroid necrosis, metaplasia, and synovial cell hyperplasia. In addition to these symptoms of osteoarthritis, some changes observed are more characteristic of rheumatoid arthritis. These changes include angiogenesis, as seen by an infiltration of red blood cells, monocytes, and macrophages. Exhibit E-C and E-D show the synovium at a higher magnification.

12. Tetracycline and their analogues are known inhibitors of MMP activity. As a result, we compared the serum levels of Dox when 1 mg/ml was added to the drinking water and the *in vitro* IC_{50}. In a MCA fluorescent assay the IC_{50} equals 59.1 μM, whereas the serum levels measured 2.64 μM using a zone of inhibition assay. These data show that the amount of Dox in the serum is 22.4 fold below the level at which 50% of MJMP activity could be inhibited. Thus, it is unlikely that there is a significant inhibition due to the Dox.

13. The unique combination of technologies described in the Specification (see, *e.g.*, page 16, lines 7-13), *i.e.*, tetracycline regulatable gene expression system and chondrocyte specific expression of a constitutively active MMP protein, has enabled development of a transgenic model resulting in lesion formation and other osteoarthritis pathologies. Line 6, which expressed significant amounts of hMMP13*, showed osteoarthritis pathologies including lesion formation, cartilage degradation, and an inflamed synovium after

five months off Dox.

14. As observed in the line 6 transgenic, the joint destruction/erosion, lesions, fibroid necrosis, metaplasia, synovial cell hyperplasia and an inflamed synovium (in the absence of T-cells) are among pathologies observed in patients with osteoarthritis. However, not all of the pathologies observed in the transgenics are reminiscent of osteoarthritis. For example, angiogenesis and infiltration of monocytes and macrophages are pathologies observed during the inflammation process associated with rheumatoid arthritis. Note, the absence of neutrophils in the synovial fluid. Migration of neutrophils to the site of inflammation is a hallmark pathology of rheumatoid arthritis.

15. These data provide direct evidence that MMP13 is a critical player in the development of osteoarthritis. Moreover, the transgenics of this invention clearly provide an animal model to test the efficacy of therapeutics. Compounds that modulate the activity of MMP13 or inhibit progression of osteoarthritis can be monitored by determining lesion formation and other osteoarthritis pathologies at various times during the progression of the disease. Finally, the fact that the rat collagen II promoter drives expression in chick chondrocytes, bovine chondrocytes and transgenic mouse joints, combined with the fact that there is no expression from this promoter in either mouse embryonic fibroblasts or HeLa cells, demonstrates that the promoters of the invention can be used for expression in a tissue specific manner in a number of different mammalian chondrocytes (and even in avian chondrocytes) in the generation of the transgenic non-human mammals of the invention.

16. I further declare that all statements made herein of my own knowledge are

true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the application or any patent issued thereupon.

Lisa A Neuhold

LISA A. NEUHOLD, Ph.D.

Dated: Princeton, New Jersey
4/6, 1999

Lisa Ann Neuhold

Wyeth-Ayerst Research
Princeton, NJ 08543-8000
Phone: (732) 274-5486
FAX No.: (732) 274-5495

3 Carey Street
Hopewell, NJ 08638
Phone: (609) 818-1610

Citizenship USA

Education

1985-1990 Ph.D., Zoology (Molecular Biology), University of Maryland, College Park, Maryland

1977-1981 B.S., Zoology, University of Maryland, College Park, Maryland

Research Interest

Development of animal models and target identification for drug discovery; Transgenic/knock-out technologies; Transcriptional profiling including Chips and RADE; Gene Expression; Arthritis; Apoptosis; Myogenesis

Research Positions

8/97-Present Senior Scientist I, Molecular Genetics Division, Wyeth-Ayerst Research, Princeton, New Jersey

8/94-8/97 Research Scientist III, Molecular Genetics Division, Wyeth-Ayerst Research, Princeton, New Jersey

7/90-7/94 Postdoctoral Fellow (Laboratory of Dr. Barbara Wold), Division of Biology, California Institute of Technology, Pasadena, California

2/85-6/90 Graduate Student, Laboratory of Developmental Pharmacology (D. Nebert; Chief), National Institute of Child Health and Human Development, National Institute of Health, Bethesda, Maryland

2/82-12/84 Research Assistant, Molecular Genetics Department, Genex Corporation, Gaithersburg, Maryland

Teaching Experience

1/89-5/89 Teaching Assistant in Cell Biology and Physiology, University of Maryland

Awards and Fellowships

1999 TACE Discovery Team Award (AHP Award)

1998 Exceptional Achievement Award (AHP Award) for Osteoarthritis Transgenic Mouse

11/91-7/94 National Institutes of Health Fellowships: National Institute of Arthritis and Musculoskeletal and Skin Diseases (Award number: AR08203-02)

7/90-7/91 Howard Hughes Fellowship

Summary of Research Activities

Wyeth-Ayerst Research

During my tenure at Wyeth-Ayerst Research, I researched and familiarized myself with the key therapeutic areas, focusing on the need for an osteoarthritis animal model for the Oncology Division. As a result, I designed, developed, and supervised production of a line of transgenic mice that exhibits experimentally regulatable cartilage degradation and lesions resembling osteoarthritis (OA).

I am collaborating with Infectious Diseases to develop an in vivo model for screening inhibitors of CMV protease activity.

I am also collaborating with the CNS Division to develop animal models of neurodegenerative disorders.

Postdoctoral Research

My research at Caltech was done in the area of myogenesis where I studied the effects of basic helix-loop-helix (bHLH) transcription factors on cellular proliferation/differentiation. Briefly, bHLH class transcription factors bind DNA *in vitro* as hetero- and homodimers. In murine myogenic cells an HLH network is composed of multiple members of the E2, MyoD and Id families; changes in the network that lead to different heteromer pools occur upon muscle determination and differentiation and have been proposed as causal. To further dissect the network and test the importance of HLH partner choice in these cellular decisions, I developed a strategy in which the identity of a bHLH dimer is molecularly specified by joining two monomers *via* a flexible polypeptide linker. The MyoD~E47 polyprotein avidly bound the same DNA target as its unlinked counterpart but, unlike intermolecular dimers, was resistant to challenge by the negative regulator Id. In cells, MyoD~E47 remained linked, prematurely activated differentiation reporters under high growth factor conditions, and displayed a complex response to excess Id, leading to a refined molecular model of HLH roles in muscle differentiation.

Graduate Research

During my graduate career at the NIH, I worked in collaboration with Dr. Keiko Ozato on the regulation of the cytochrome P_1450 promoter. Briefly, the mouse cytochrome P_1450 gene is responsible for the metabolism of numerous carcinogens and toxic chemicals. Induction by the environmental contaminant tetrachlorodibenzo-*p*-dioxin (dioxin) requires a functional aromatic hydrocarbon (Ah) receptor. The focus of my research was to identify those DNA binding elements responsible for the activity of the cytochrome P_1450 promoter. Deletion analysis on the 5' flanking region of this gene revealed two *cis*-acting elements that were essential for both dioxin inducible and constitutive activity. Subsequently, biochemical analysis allowed me to characterize the DNA binding proteins into three classes and identify their DNA binding sites.

Prior to my graduate studies, I worked at Genex Co. on projects involving the expression of heterologous proteins (*i.e.*, interleukin-2, antithrombin-3, etc.) in *E. coli*.

Summary of Research Skills

Preparation of plasmid and genomic DNA; design/construction of prokaryotic expression vectors; cloning; bacterial protein expression systems; colony hybridizations; design/construction of eukaryotic expression vectors for transgenic mice; southern blots; PCR cDNA cloning; PCR-based genotyping of transfected cell lines; restriction mapping; PCR analysis; PCR-based subcloning; purification and radiolabelling of oligonucleotides; site-directed mutagenesis; sequencing; *in vitro* transcription/translation systems; gel electrophoresis; SDS-polyacrylamide protein gel; transient and stable gene transfection/transformations; isolation/characterization stable transformants; design/construction of mammalian and viral expression vectors; growth and preparation of monoclonal antibodies from hybridoma cells; immunocytochemistry using monoclonal antibodies; protein assay; CAT assays; b-galactosidase assays; luciferase assays; preparation of nuclear/cellular protein extracts from mammalian cells and mouse tissues; mobility shift assays; methylation interference; DNA footprinting; western blots; protein activity assays, *i.e.*, zymograms. Experience with preparation of total RNA from both mammalian cells and mouse tissues; northern blots; *in vitro* transcription/ribonuclease mapping; RT-PCR; Taqman quanitative PCR; nuclear run-off experiments; ligation mediated PCR *in vivo* footprinting; non-radioactive detection systems; Knowledge of IBM and Macintosh computer software.

Publications

Neuhold, L. A., Gonzalez, F. J., Jaiswal, A. K., and Nebert, D. W. (1986). Characterization of the dioxin-receptor complex-binding site in the upstream sequences of the mouse P1450 gene and interaction with heterologous SV40 promoter. *DNA* **5**, 403-411.

Jaiswal, A. K., Neuhold, L.A., and Nebert, D. W. (1987). Human P450IA1 upstream regulatory sequences expressing the chloramphenicol acetyltransferase gene. Effect of HaMSV enhancer and comparison of transient with stable transformation assays. *Biochem. Biophys. Res. Commun.* **148**, 857-863.

Neuhold, L. A., Shirayoshi, Y., Ozato, K., Jones, J. E., and Nebert, D. W. (1989). Regulation of the mouse CYP1A1 gene expression by dioxin. Requirement of two *cis*-acting elements during the induction process. *Mol. Cell. Biol.* **9**, 2378-2386.

Carrier, F., Owens, R. A., Neuhold, L. A., Nebert, D. W., and Puga, A. (1992). Activation of the murine *Cyp1a-1* (cytochrome P_1450) gene requires protein phosphorylation: Possible involvement of protein kinase C, p. 449-451. In: *Cytochrome P-450 Biochemistry and Biophysics* (A. I. Archakov and G. I. Bachmanova, Editors). Joint Stock Co.

Neuhold, L. A., and Wold, B. (1993). HLH forced dimers: E47/ITF1 tethered to MyoD is an active myogenic DNA binding factor and is insulated from negative regulation by Id. Cell **74**, 1033-1042.

Abstracts

Neuhold, L. A., and Wold, B. (1993). MyoD tethered to ITF-1 activates a muscle-specific enhancer in undifferentiated myoblast. Abstract: Keystone Symposia on Molecular and Cellular Biology. *J. Cell. Biochem.*, Supplement 17A, 203.

References

Louis DeGennaro, Ph.D.
Director
Molecular Genetics Division
Wyeth-Ayerst Research
Princeton, NJ 08543-8000
Phone: (732) 274-4002

Loran Killar, Ph.D.
Associate Director
Oncology Division
Wyeth-Ayerst Research
Princeton, NJ 08543-8000
Phone: (732) 274-4002

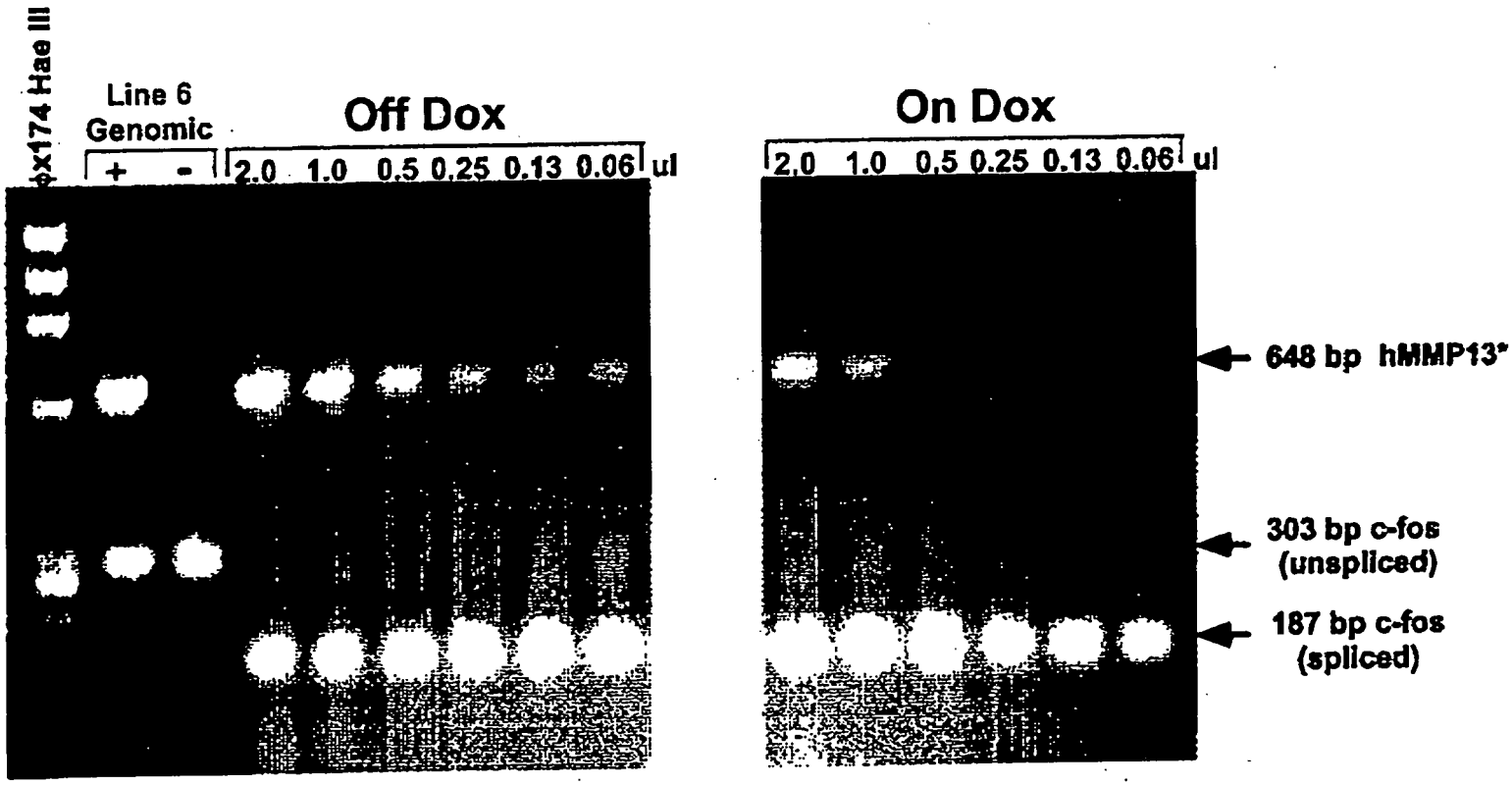
Barbara Wold, Ph.D.
Associate Professor,
Division of Biology, 156-29
California Institute of Technology
Pasadena, California 91125
Phone: (818) 395-4916

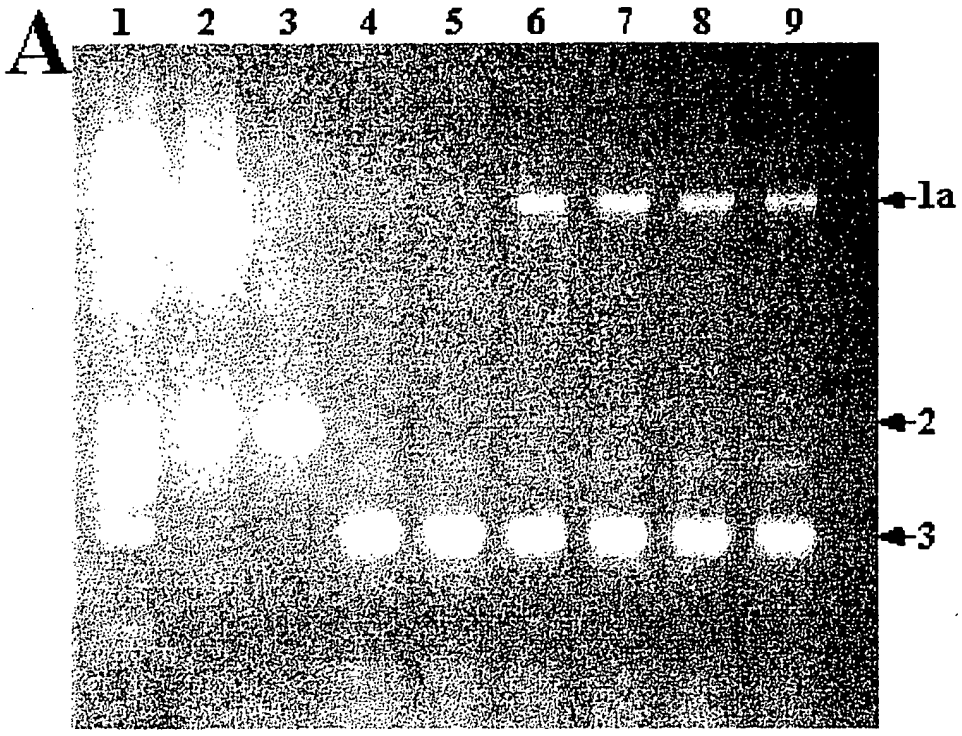
Norman Davidson, Ph.D.
Norman Chandler Professor of Chemical Biology, Emeritus
Division of Biology, 156-29
California Institute of Technology
Pasadena California 91125
Phone: (818) 395-6055

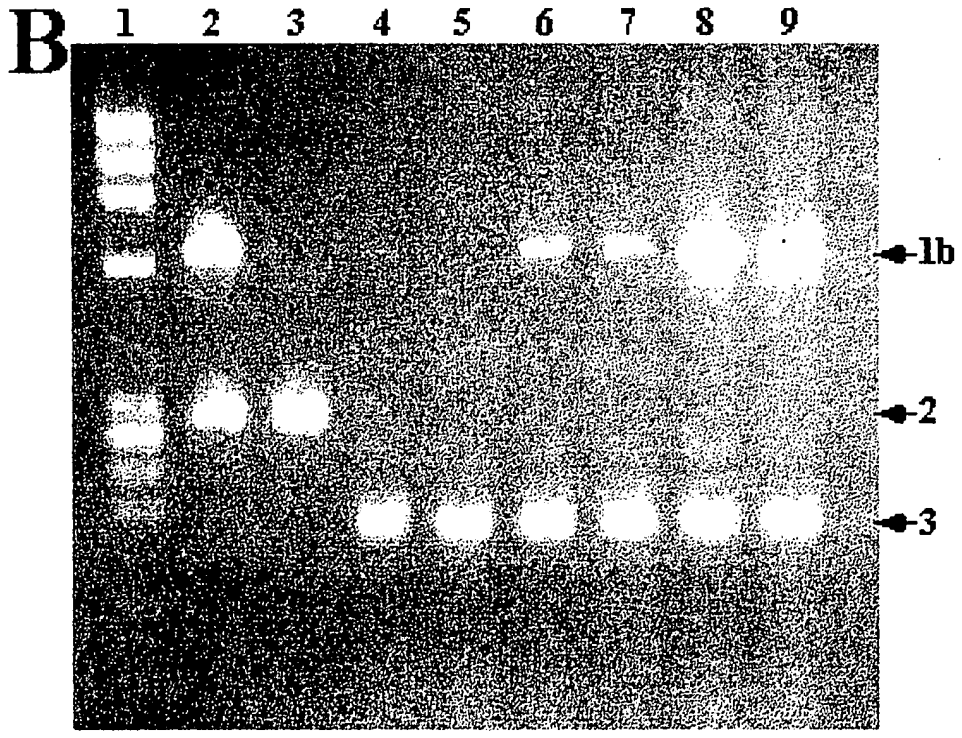
Keiko Ozato, Ph.D.
Section Chief, Laboratory on Developmental and Molecular Immunity
National Institute of Child Health and Human Development
Building 6, 2A01
National Institutes of Health
Bethesda, Maryland 20892
Phone: (301) 496-9184

Thomas D. Sargent, Ph.D.
Section Chief, Laboratory on Vertebrate Development
National Institute of Child Health and Human Development
Building 6B, Room 4B-412
National Institutes of Health
Bethesda, Maryland 20892
Phone: (301) 496-0369

Line 6: Titration of hMMP13* by RT-PCR







Expression profile of the TA and MMP13 RNA by RT-PCR. (A) amplification of TA cDNA from total RNA. (B) amplification of MMP13* cDNA from total RNA. Lane 1: ϕx174 Hae III MW markers; Lane 2: PCR amplification of transgenic (line 6) genomic DNA; Lane 3: PCR amplification of non-transgenic genomic DNA; PCR amplification of transgenic (line 6) genomic DNA; Lanes 4: a wild-type mouse maintained on Dox; Lanes 5: a wild-type mouse off Dox; Lanes 6-7: transgenic mice (~4 mos) maintained on Dox; Lane 8-9: transgenic mice (~4 mos) removed from Dox at birth. The arrows 1a and 1b indicate a 648 bp MMP13* specific fragment and a 859 bp specific fragment, respectively. Each reaction was run using c-fos primers as an internal control, spliced mRNA yielding 187 bp (arrow 3) and unspliced mRNA 303 bp (arrow 2). Note, no bands were detected in corresponding lanes containing RNA for PCR that was not treated with RT (data not shown).